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A Novel B7-2 (CD86) Splice Variant with a Putative Negative Regulatory Role

Efstathia K. Kapsogeorgou, Haralampos M. Moutsopoulos, and Menelaos N. Manoussakis

B7-2 (CD86) costimulatory molecules are pivotal for the regulation of T cell responses. In this study, a novel human B7-2 alternate transcript (termed B7-2C) is described. This transcript is characterized by the deletion of exon 4 that encodes the IgV-like counter-receptor binding domain of the B7-2 protein (full-length; B7-2A). B7-2C was detected as mRNA and cell surface protein in human non-neoplastic salivary gland epithelial cells and monocytes, but not in fibroblasts, T cells, B cells, dendritic cells, and several epithelial tumor cell lines. In monocytes, B7-2C protein expression was found to be significantly down-regulated following activation. The analysis of Chinese hamster ovary (CHO) single-transfected (CHO-B7-2C) and double-transfected (CHO-B7-2A/B7-2C) cell lines had indicated that cell surface B7-2C expression is by itself unable to provide T cell costimulation, but inhibits the transmission of costimulatory signals via B7-2A (by 23–69%). Such inhibition was found to depend on the relative cell surface expression of B7-2A and B7-2C proteins, as it occurred in CHO-B7-2A/B7-2C transfecants with significantly lower B7-2A to B7-2C ratios (1.0–3.5), compared with those with unaffected B7-2A-mediated costimulatory function (10.0–19.5). Our findings suggest that B7-2C is expressed by monocytes, as well as by nonimmune cells with potential Ag-presenting capacity (such as salivary gland epithelial cells). The expression of B7-2C on certain B7-2A-expressing cells appears to represent a mechanism for the fine tuning of B7-2A-mediated costimulatory signals, possibly through the interruption of B7-2A clustering required for the productive interaction between B7-2A and cognate receptors.


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mRNA splicing variant by SGECs, as well as by peripheral blood monocytes. This transcript (named B7-2C) is characterized by the deletion of exon 4, which encodes the IgV-like counter-receptor binding domain of the B7-2 protein. We show that B7-2C encodes a surface protein, whose expression is diminished during activation. Furthermore, we present evidence that the truncated B7-2C protein negatively regulates the costimulatory function of the full-length B7-2. These findings further support the complexity of the B7 costimulatory pathways and the implication of these molecules in the fine tuning of immune responses.

Materials and Methods

Reagents

The mAbs against human B7-2 (clone FUN.1), CD28 (clone CD28.2), CD3 (clone HIT3a), CD14 (clone M5E2), HLA-DR (clone G46-6), CD83 (clone HB15e), and CD1a (clone MT102) were purchased from BD Pharmingen. The mAbs against human CD4 (clone SK5) and CD8 (clone SK1) were obtained from BD Biosciences. The rabbit polyclonal Ab 19 that is raised against the cytoplasmic region of B7-2 protein was from Santa Cruz Biotechnology. The mAb to human cytokeratins 5, 6, 8, 17, and 19 (clone MNF116) and to dendritic reticulum cell (clone R4/23) was obtained from DakoCytomation. The mAb (clone 9B11) that recognizes the tag peptide myc, which is fused at the cytoplasmic terminal of the B7-2 protein form, was from Santa Cruz Biotechnology. The mAbs against human IL-2Rα, CD25, 7G7B6), CD20 (clone 2H7), and the CD28-Ig fusion protein (human CD28 protein fused with mouse Ig) were purchased from Ancell. The CTLA4-Ig fusion protein (human CTLA4 protein fused with human Ig) was a gift from RepliGen. PE-conjugated F(ab')2 against mouse and human IgG Fe fragments were from Rockland. For use in costimulation assays, mAbs were bound to sheep anti-mouse IgG-coated Dynabeads M450 (Dynal Biotech) at a concentration of 1 mg of mAb/10⁷ beads, according to the manufacturer's instructions. Peptidoglycan (PGN) from Staphylococcus aureus was purchased from Sigma–Aldrich. GM-CSF, IL-4, TNF-α, and IL-1β were obtained from R&D Systems. IFN-α was from Scherping-Plough.

Cell lines

Non-neoplastic, long-term cultured SGEC lines and salivary gland fibroblasts were established from minor salivary gland biopsy samples obtained with informed consent from patients undergoing diagnostic evaluation for possible Sjögren’s syndrome, as previously described (16). The purity and epithelial origin of cultured SGEC lines was routinely verified by morphological features, proliferation rate or survival (16). However, SGEC lines obtained from patients with Sjögren’s syndrome have been shown to display a constitutively activated phenotype as suggested by the significantly increased spontaneous expression of several immunomodulatory molecules (including cytokines, chemokines, TLRs, and MHC, costimulatory, and adhesion molecules) compared with control SGEC lines from patients not found to have Sjögren’s syndrome (17). Regarding B7-2 molecules, constitutive expression of the full-length B7-2 mRNA and protein is detectable in all SGEC lines (15). However, expression levels vary considerably among cell lines, ranging from low to high (albeit the latter are significantly lower compared with those of B cells, monocytes, and dendritic cells (DC)). High levels of expression are typically observed in the constitutively activated SGEC lines of patients with Sjögren’s syndrome (17).

The neoplastic epithelial cell lines HeLa (cervical adenocarcinoma), PC3 (prostate), and MDA (mammary) were maintained in DMEM (Invitrogen Life Technologies) supplemented with 10% FBS (Invitrogen Life Technologies), 2 mM t-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin.

The Jurkat T cell line and the EBV-transformed B cell line JY were cultured in RPMI 1640 supplemented with 10% FBS, 5 × 10⁻⁷ M 2-ME, 2 mM t-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin. All cultures were incubated at 37°C, 5% CO₂, and 99% humidity.

Isolation of peripheral blood subpopulations

PBMC from healthy donors were separated by density-gradient centrifugation on Ficoll-Paque (Amersham Biosciences). B cells were isolated by immunomagnetic positive selection using anti-CD19-coated Dynabeads M450 (Dynal Biotech). B cell purity was routinely >98%, as determined by flow cytometric analysis using FITC-labeled anti-CD20 mAb. CD4⁰ T cells were purified from T cell-enriched PBMC by immunomagnetic positive selection using Dynabeads M450-coated with anti-CD4 (Dynal Biotech), as previously described (15). By flow cytometry, the purity of CD4⁺ T cell population yields was routinely >99.5%, and the isolated CD4⁺ T cells showed minimal expression of CD25 (IL-2Rα, <5% positive), whereas they were unresponsive to stimulation by anti-CD3 mAb only. Monocyte-enriched population was isolated from PBMC by immunomagnetic negative selection (Dynal Biotech), according to manufacturer’s instructions. This population consisted of >90% CD14⁺ monocyte cells by flow cytometry and were used as nonstimulated and following activation by treatment with PGN (100 μg/ml) for 8 h (early activation) and 24 h (late activation).

Preparation of monocytoid DCs

Immature monocytoid DCs were prepared from isolated peripheral blood monocytes after cultivation in fresh complete RPMI 1640 medium supplemented with 1000 U/ml GM-CSF and 25 ng/ml IL-4 for 7 days. The maturation of DCs was induced by treatment with 20 ng/ml TNF-α, 10 ng/ml IL-1β, and 500 U/ml IFN-α for 4 days. DCs were routinely analyzed for the expression of HLA-DR, B7-2, CD14, and CD83 molecules by flow cytometry.

Characterization of B7-2 mRNA molecules

Total RNA was extracted with the RNasey mini kit (Qiagen). The 1 μg of RNA was reversed transcribed using oligo(dT) primers (MWG Biotechology) and ImProm-II reverse transcriptase (Promega). The integrity of all cDNA samples was tested by RT-PCR for β-actin mRNA (15). The amplification of the entire coding region of the B7-2 mRNA was accomplished by previously described (11) with slight modifications to the proofreading Pfu polymerase (Stratagene). In brief, the PCR included an initial denaturation step at 94°C for 4 min, 35 cycles of 94°C for 1 min, 56°C for 1 min, and 72°C for 2 min followed by a final extension step at 72°C for 5 min. The amplified fragments were size-separated in 1% agarose gel and visualized by ethidium bromide. Subsequently, the PCR products were isolated by the QIAquick gel extraction kit (Qiagen) and identified by automated sequencing (ITE; IBMM and MWG Biotechnology). The PCR products that were isolated from SGEC corresponded to the transcripts that encode for the full length (13, 14), the soluble form of the B7-2 protein (11), and a novel alternate B7-2 variant that lacks exon 4 (hereafter referred as B7-2A, B7-2B, and B7-2C, respectively).

CHO cell lines stably transfected with B7-2 transcripts

CHO cell lines transfected with the full-length B7-2 (CHO-B7-2A), and mock-transfected CHO cell lines (CHO-mock) were provided by V. Bousios (Dana-Farber Cancer Institute, Boston, MA) and were maintained in CHO medium supplemented with the selection antibiotic geneticin (G418, 400 mg/ml; Invitrogen Life Technologies). B7-2C cDNA was cloned in the pcDNA6/myc-His-B expression vector (Invitrogen Life Technologies), which enabled the fusion of 10 aa myc tag to the cytoplasmic terminal of the B7-2 protein. This construct or the pcDNA6/myc-His-B vector alone were transfected to CHO cells, as well as to CHO-B7-2A and CHO-mock transfectants using the Gene Jammer transfection reagent (Stratagene), according to the manufacturer’s recommendations. Stably transfected clones were selected and maintained by cultivation in CHO medium containing the appropriate selection antibiotics blasticidin (10 mg/ml; Invitrogen Life Technologies) or geneticin. The expression of B7-2C protein in B7-2C-transfected clones was routinely verified in total cytoplasmic extracts by immunoblotting against the myc tag. This approach resulted to the establishment of CHO cell lines that expressed B7-2C or B7-2A proteins, as well as the respective mock transfectants. In detail, these CHO cell lines were the following: CHO-B7-2C and CHO-mock (CHO cells transfected with B7-2C transcript or the pcDNA6/myc-His-B vector, respectively); CHO-B7-2A/CHO-B7-2C (CHO-B7-2A/C) and CHO-B7-2A/mock (CHO-B7-2A cells transfected with the B7-2C transcript or the pcDNA6/ myc-His-B vector, respectively); and CHO-mock/B7-2C and CHO-mock/ mock (CHO-mock transfected with the B7-2C transcript or the pcDNA6/ myc-His-B vector alone, respectively).
Identification of a novel alternate transcript of the human B7-2 costimulatory molecule (B7-2C) that encodes a cell surface protein. A, Representative example of agarose gel electrophoresis showing the three B7-2 products (named B7-2A, B7-2B, and B7-2C) that are detected in human non-neoplastic SGEC by RT-PCR specific for the entire B7-2 mRNA coding region. As revealed by sequencing analysis (three distinct SGEC lines), these PCR products represent distinct splicing variants of the B7-2 costimulatory molecule, whose organization is displayed in the schematic representation. The exons (indicated by numbers) that encode each protein domain of the B7-2 protein. (Cyt.R, cytoplasmic region; IgC, IgC-like region; IgV, IgV-like region; SP, signaling peptide; and TrM transmembrane region are represented (shaded)). B7-2A and B7-2B products correspond to the previously described variants that encode the full-length and the soluble form of the B7-2 protein, respectively, whereas B7-2C is a novel B7-2 transcript. B7-2C is characterized by the deletion of exon 4 (nt 47 to 382) that encodes the counter-receptor binding IgV-like domain of the B7-2 protein. B, Representative demonstration of the B7-2 mRNA variants expression by CHO-mock/mock (m/m), CHO-B7-2A/mock (A/m), and CHO-B7-2A/C transfectants, SGEC displaying high (H) or low (L) surface expression of B7-2A (as revealed by flow cytometry) salivary gland fibroblasts (SGF), CD4

**Western blotting**

Protein extracts were prepared from transfected CHO cell lines and the expression of B7-2 protein forms was analyzed by SDS-PAGE and immunoblotting, as previously described (15). Specific Abs and the ECL system CDP-Star (Roche) were applied for the protein detection by immunoblotting (15). B7-2C proteins were detected by an Ab specific for the myc tag fused at the cytoplasmic terminal of the protein or the C19 polyclonal Ab that recognizes the cytoplasmic region of the B7-2 protein, which is conserved in the B7-2C form. The full-length B7-2A molecule was identified by the FUN.1 mAb that recognizes the IgV-like domain of the B7-2 protein.

**Detection of cell surface protein expression by biotinylation of surface proteins and immunoprecipitation**

The expression of the B7-2C protein on the cell surface was evaluated by a qualitative method that combines the biotinylation of the cell surface proteins, followed by immunoprecipitation with specific Abs. The cell surface proteins were biotinylated by sulfo-NHS-biotin (Pierce), according to the manufacturer’s recommendations. Briefly, CHO transfectants (3 × 10^6 cells), nonstimulated or activated peripheral blood monocytes (10 × 10^6 cells) or SGEC (5 × 10^6 cells) were harvested, counted, washed three times with ice-cold PBS (pH 8.0) and incubated with sulfo-NHS-biotin solution (1 mg of biotin/ml; CHO and monocytes; 25 × 10^6 cells/ml; SGEC: 10 × 10^6 cells/ml of biotin solution) for 30 min at 4°C. The biotinylation reaction was quenched by the addition of serum-free RPMI and the excess of biotin was removed by washing cells five times with PBS. The labeled cells were lysed as previously described (15) and B7-2 proteins were selectively immunoprecipitated by FUN.1 mAb (full-length B7-2A), anti-myc mAb (myc-tagged truncated B7-2C; transfected CHO cells), or C19 polyclonal Ab (both B7-2A and B7-2C protein forms). Briefly, precleared protein extracts were incubated with the appropriate Abs (3 µg Ab/sample; 2-h incubation) and subsequently the protein-Ab complexes were precipitated by protein G-immobilized on Sepharose-4B (overnight incubation; Sigma-Aldrich). The immunoprecipitated proteins were eluted by boiling in SDS sample buffer and analyzed by SDS-PAGE. The biotin-labeled precipitated proteins (originating from the cell surface) were detected by immunoblotting using alkaline phosphatase-conjugated streptavidin and the CDP-Star ECL system.
were biotinylated and the B7-2A and B7-2C protein forms were concurrently immunoprecipitated by the C19 Ab. The density of the biotin-labeled B7-2A and B7-2C protein bands expressed on each cell line was estimated by the ImageQuant Software (Molecular Dynamics), and the ratio of the cell surface expression of these proteins was calculated. Three independent experiments were performed for each cell line. Preliminary immunoprecipitation experiments using CHO-B7-2A/C cells indicated that C19 Ab does not preferentially bind to one of the two B7-2 protein forms, as demonstrated by similar B7-2A to B7-2C ratios to those obtained using separate Abs specific for each isoform (FUN.1 and anti-ιννε, respectively).

Flow cytometry

For the analysis of the surface B7-2A proteins, ~5 × 10⁴ cells were stained either directly using FITC- or PE-conjugated mAbs and isotype controls or indirectly by fusion proteins (CD28-Ig or CTLA4-Ig, 50 μg/ml) followed by incubation with the appropriate PE-conjugated IgG Fc fragment–specific secondary Abs, as previously described (15). Analyses were performed using the FACSCalibur flow cytometer and CellQuest software (BD Biosciences). Mean fluorescence intensity values obtained by staining with specific mAbs were corrected by the subtraction of background values (isotype control mAbs).

Costimulatory activity of the CHO transfectants

The capacity of the established CHO cell lines to costimulate the proliferation of anti-CD3-activated CD⁴⁺ T cells by coated magnetic beads was assessed in standard costimulation assays, as previously described (15). In brief, confluent cultures of CHO transfectant cells were mildly fixed with 1% paraformaldehyde for 10 min in 96-well microtiter plates (Costar) and received CD⁴⁺ T cells (2 × 10⁵ cells/well, 200 ml/well in complete RPMI 1640) in the presence or absence of anti-CD3-coated magnetic beads (three beads per CD⁴⁺ cell), in triplicates for each combination. The proliferative responses of CD⁴⁺ T cells were estimated after 3 days of culture by [³H]thymidine incorporation. Cells were pulsed with [³H]thymidine (1 mCi/well; ICN) for the last 18 h of culture. Subsequently, cells were collected onto glass fiber filters (Molecular Devices), and the incorporation of radioactivity was measured in a liquid scintillation analyzer (Packard Instruments).

To evaluate the effect of B7-2C expression on the costimulatory function of the full-length B7-2A protein, pairs of CHO-B7-2A/mock and CHO-B7-2A/C cell lines were selected on the basis of similar surface expression of the full-length B7-2A protein (as assessed by flow cytometry) and were assayed for their ability to costimulate the proliferation of anti-CD3-activated CD⁴⁺ T cells in standard costimulation assays. The costimulatory activity of the paired cell lines was compared and in certain CHO-B7-2A/C cell lines was found reduced. In each pair of CHO transfectants, the percentage of the costimulated proliferation reduction was calculated as (cpm of CHO-B7-2A/mock – cpm of CHO-B7-2A/C)/(cpm of CHO-B7-2A/mock) × 100. Three independent experiments were performed for each pair of cell lines and the median reduction (range) was estimated.

Results

Identification of a novel alternate B7-2 transcript that lacks exon 4, which encodes the IgV-like domain

In humans, B7-2 mRNA consists of eight exons in which the region of exon 3 to exon 8 encodes the full-length protein (18). By RT-PCR that was specifically designed to amplify the entire coding region of the B7-2 mRNA (exons 3 to 8), three distinct alternate transcripts of the B7-2 mRNA molecules were detected in cultured non-neoplastic SGECs (Fig. 1A). In agreement to our previously published data showing the expression of membrane-bound and soluble B7-2 proteins by SGECs (15), the sequencing analysis revealed that the first two transcripts were identical to the formerly described variants that encode the full-length form and the soluble form of the B7-2 protein (972 and 828 bp, designated in this study as B7-2A and B7-2B, respectively) (Fig. 1A) (11, 13, 14). The third product, named B7-2C, represents a novel splice variant of the B7-2 costimulatory molecule that has a deletion of nt 47 (starting from ATG codon) to 382 (Fig. 1A). According to the cd86 genomic organization (18), the deleted region of the B7-2C transcript corresponds exactly to the exon 4 that encodes the counter-receptor IgV-like binding domain of B7-2 protein. In this transcript, the deletion of exon 4 did not affect the open reading frame, whereas the sequences of the remainder exons that encode the signaling peptide, the IgC-like, the transmembrane and the cytoplasmic region of the B7-2 protein were completely preserved (Fig. 1A). In accordance with our previous results (15), the expression of B7-2A transcript was detected in all SGEC lines examined (n = 21), whereas 17 of them also expressed the B7-2B and 10 the B7-2C splice variant. B7-2C mRNA expression was found confined in SGEC lines characterized by low constitutive surface expression of the full-length B7-2A protein, but not in SGECs with high constitutive surface B7-2A expression, as illustrated by flow cytometry experiments (Fig. 1B and data not shown). The expression of the B7-2C variant was also investigated in several other cell types (non-neoplastic, neoplastic, or transformed cell lines). Apart from non-neoplastic SGECs, B7-2C was also detected in nonstimulated peripheral blood monocytes, but not in salivary gland fibroblasts, peripheral blood T and B lymphocytes, immature or mature monocytoid DCs, Jurkat T cells, EBV-transformed B cells, or certain neoplastic epithelial cell lines (Fig. 1B). The expression of either B7-2B (that encodes the soluble form) or B7-2C

![FIGURE 2. The expression of B7-2C molecules by cultured non-neoplastic SGEC was found to associate with low surface expression of the full-length B7-2A protein. A, Representative examples of SGEC lines manifesting high (left) or low (right) constitutive surface expression of the B7-2A protein, as assayed by flow cytometry using a PE-conjugated anti-B7-2 mAb (FUN.1) (open histogram). Staining with an isotype-control Ab (negative control) (gray-shaded histogram) is also shown. B, Immunoblotting analysis of surface expression of the B7-2A and B7-2C protein isoforms on representative SGEC lines manifesting high or low constitutive surface B7-2A protein expression (as revealed by flow cytometry). SGEC lines used are the same as shown in A. Surface proteins of cells were biotinylated, and B7-2 proteins were subjected to immunoprecipitation using the anti-cyttoplasmic B7-2 Ab C19 (that recognizes the cytoplasmic tail of both B7-2A and B7-2C isoforms). An irrelevant Ab (Ct. Ab) was applied for the evaluation of nonspecifically immunoprecipitated proteins (negative control). Immunoprecipitated proteins were analyzed by SDS-PAGE, and biotinylated molecules were visualized by immunoblotting with alkaline phosphatase-conjugated streptavidin. Among SGEC lines with high and those with low constitutive surface B7-2A expression, significantly higher constitutive expression of B7-2C proteins was detected in SGECs with low B7-2A expression. Compared with B7-2A and B7-2C protein isoforms expressed by CHO-B7-2A/C transfectants, those of SGEC correspond to glycoprotein bands of higher molecular masses, compatible with heavier glycosylation status. The experiments performed are representative of three SGEC lines from each group (high and low B7-2A expression, respectively). CHO-mock/mock (m/m) and CHO-B7-2A/C transfectants were used as negative and positive control cell lines, respectively. An irrelevant protein band that is detected by the alkaline phosphatase-conjugated streptavidin reagent (asterisk), which was applied for the visualization of biotinylated proteins.](http://www.jimmunol.org/content/165/10/3818/F1.large.jpg)
transcripts was found to be restricted in cell lines that express the full-length B7-2A molecule (Fig. 1B).

B7-2C transcript encodes a protein that is expressed on the cell surface and is negatively regulated by activation processes

B7-2C transcript is expected to encode a 245 aa protein. This transcript apparently encodes a membrane protein, as suggested by the conservation of the open reading frame, the signaling peptide and the transmembrane region. Most likely due to their reactivity with the IgV-like domain, commercially available monoclonal and polyclonal Abs to surface B7-2 protein were found unable to detect the expression of B7-2C protein on CHO-B7-2C transfectants (data not shown). Hence, to enable the B7-2C study, CHO cell lines were stably transfected with B7-2C transcript that was tagged with a myc peptide at the cytoplasmic terminal of the molecule. To investigate the membrane expression of B7-2C protein, an indirect detection method was applied. Surface proteins of CHO-B7-2C cells were labeled by biotinylation and the expression of surface B7-2C molecules was assessed by immunoprecipitation with Abs to the myc tag (anti-myc mAb) or to the cytoplasmic tail of the B7-2 proteins (C19 Ab), followed by the detection of biotinylated proteins. Both these Abs were found to immunoprecipitate multiple protein bands of molecular masses ranging from ~55 to 75 kDa (a pattern compatible with glycosylated proteins, such as B7-2 proteins), indicating the cell surface expression of the protein encoded by the B7-2C transcript (Fig. 1C). The application of the same method in nontransfected, non-neoplastic SGEC lines (n = 3) and peripheral blood monocyte lines (n = 3) revealed the expression of two proteins with molecular masses that correspond to B7-2A and B7-2C, respectively (Figs. 2 and 3). These findings further support that the B7-2C transcript encodes a membrane protein. In line with our previous findings, showing that the full-length and soluble B7-2 proteins of SGECs run at a higher range of molecular masses (a fact indicative of heavier glycosylation status) compared with EBV-transformed JY B cells (15), the B7-2C protein molecules of SGECs present higher molecular masses of the respective molecules expressed by CHO transfectants (Fig. 2).

It has been documented that B7-2A is constitutively expressed on the surface of resting monocytes and is rapidly

FIGURE 3. Surface expression of B7-2A and B7-2C protein isoforms in preparations of peripheral blood monocytes and monocytoide DCs. A, Flow cytometric analysis of the modulation of the surface B7-2A expression by peripheral blood monocytes (MNC) (left) and monocytoide DC (right) following activation and maturation, respectively, using a PE-conjugated anti-B7-2 mAb (FUN.1) (filled histograms) or an isotype control Ab (negative control staining) (open histograms). Left, Nonstimulated (NS) monocytes (thick line open histogram) display moderate amounts of surface B7-2A that are up-regulated by PGN at 8 h (light gray filled histogram), but are significantly down-regulated at 24 h of treatment (dark gray filled histogram). Right, Immature DCs (light gray filled histogram) display moderate amounts of surface B7-2A that are significantly up-regulated in mature DCs (dark gray filled histogram). B, Immunoprecipitation/immunoblotting analysis of the modulation of surface expression of the B7-2A or B7-2C protein isoforms on peripheral blood monocytes (MNC) and on monocytoide DCs following activation and maturation, respectively. Preparations used are the same shown in A and included nonstimulated (NS) monocytes, PGN-activated monocytes (for 8 and 24 h), immature DCs (Im), and mature DCs (Mat), as well as CHO-B7-2m/m (m/m) transfectants (negative control), CHO-B7-2m/C (m/C) (positive control), CHO-B7-2A/m (A/m) (positive control), and CHO-B7-2A/C transfectants (A/C) (positive control). Surface protein biotinylation, immunoprecipitation of B7-2 proteins and immunoblotting were performed as in Fig. 2B. An irrelevant Ab (Cl. Ab) was applied for the evaluation of nonspecifically immunoprecipitated proteins (negative control). Both B7-2A and B7-2C proteins are readily detected in nonstimulated monocytes. PGN activation of monocytes at 8-h results in significant up-regulation of surface B7-2A expression and down-regulation of surface B7-2C expression, whereas at 24-h of treatment monocytes manifest a modest down-regulation of surface B7-2A, but almost complete elimination of surface B7-2C expression. Similar results were obtained using monocytes from the same blood donor in an independent experiment, as well as from another healthy blood donor, in which the expression of B7-2C was found to be abolished more rapidly following activation (at 8 h of PGN activation). B7-2C appears as multiple protein bands compatible to its glycoprotein nature. The lower protein bands of B7-2C overlap with the nonspecific band (asterisk); however, the reduction of its expression is readily appreciated at the areas above the nonspecific band. Immature DCs (Im) display moderate amounts of surface B7-2A that are significantly up-regulated in mature DCs (Mat), whereas no evidence of surface B7-2C protein expression was detected in either DC preparations. The relative positions of B7-2A and B7-2C protein isoforms are also shown in CHO-B7-2A/mock, CHO-B7-2m/C and CHO-B7-2A/C transfectants, whereas an irrelevant band (asterisk) is detected by alkaline phosphatase-conjugated streptavidin.

FIGURE 4. B7-2C protein does not interact with soluble CD28 and CTLA4 fusion proteins, whereas its coexpression on B7-2A-expressing CHO transfectants does not alter B7-2A binding to CD28 and CTLA4 receptors. Representative examples of flow cytometric analyses of the binding of CD28-Ig and CTLA4-Ig fusion proteins (open histograms) on single and double CHO transfectants that expressed either the B7-2A or the B7-2C isoforms, as well as CHO-mock/mock transfectant (negative control cell line). CD28-Ig and CTLA4-Ig fusion proteins do not react with CHO-B7-2A cells, whereas their binding to B7-2A (as observed in CHO-B7-2A/mock) is not affected by the coexpression of B7-2C in B7-2A transfectants (CHO-B7-2A/C). For comparison, the detection of the B7-2A protein expression by a PE-conjugated anti-B7-2 mAb (FUN.1) (open histograms) is shown. In all experiments, staining with an isotype control Ab served as a negative control (light gray filled histograms).
up-regulated following activation. To investigate the expression kinetics of the truncated B7-2C protein and its relative expression to the full-length B7-2A, the surface expression of both forms was assayed in nonstimulated and activated (for 8 and 24 h) peripheral blood monocytes. In preliminary experiments, PGN (100 μg/ml) was found the most potent inducer of the full-length B7-2A surface expression, compared with other activators tested (such as LPS or polyinosinic-polycytidylic acid). As shown in Fig. 3A (left), treatment for 8 h with PGN resulted in the induction of the surface B7-2A expression by monocytes, whereas longer activation (24-h treatment) led to significant down-regulation of its expression compared with the constitutive expression by nonstimulated monocytes. In contrast, B7-2C surface expression was found to be reduced at 8 h of activation and radically decreased or completely eliminated following activation for longer period (24 h) (Fig. 3B). As a consequence, significantly increased ratios of surface-expressed B7-2A to B7-2C proteins were noticed in preparations of monocytes activated for 8 h (median ratio: 30.5, range: 14.9 to 46.2) compared with ratios from nonstimulated monocytes (median ratio: 2.4, range: 2.0 to 2.8). Due to diminished B7-2C expression, the ratio of the surface-expressed B7-2A to B7-2C proteins was further increased (ratio greater than 169.0) on 24 h-activated monocytes, despite the reduction on surface B7-2A protein expression. Moreover, activation of monocytes by 2-h adherence in plastic was also found to down-regulate the surface B7-2C expression (data not shown), suggesting that B7-2C is subjected to negative regulation by various activation procedures.
B7-2C protein inhibits the costimulatory function of the full-length B7-2A molecule

B7-2C isoform is expected to be unable to interact with counter-receptors due to the lack of the counter-receptor binding IgV-like domain. In line with this expectation, B7-2C-expressing CHO transfectants were not stained by CD28-Ig or CTLA4-Ig fusion proteins in flow cytometric analyses (Fig. 4) and were found unable to costimulate the proliferation of anti-CD3 activated CD4⁺ T cells in standard costimulation assays (Fig. 5C).

To investigate possible interactions between the truncated B7-2C and the full-length B7-2A, stable double-transfected CHO cell lines that express the B7-2A protein with or without the truncated form B7-2C (designated as CHO-B7-2A/C and CHO-B7-2A/mock, respectively) were established. Preliminary analyses in CHO-B7-2A/C transfectants indicated that the expression of the B7-2C transcript did not affect the surface expression of the B7-2A protein. Furthermore, flow cytometric analyses using CD28-Ig or CTLA4-Ig fusion proteins revealed that B7-2C expression did not alter the binding of the B7-2A protein to its receptors (Fig. 4). In addition, B7-2A and B7-2C proteins were not found to heterodimerize, as supported by the lack of coimmunoprecipitation of B7-2A and B7-2C molecules by specific Abs to either protein form (FUN.1 or anti-myc, respectively) in CHO-B7-2A/C cells (data not shown). To examine whether B7-2C interferes with the function of the full-length B7-2A protein, comparative analyses of the costimulatory capacity were performed in CHO-B7-2A/C and CHO-B7-2A/mock cell lines (n = 12 pairs), as selected on the basis of similar surface B7-2A protein expression (Fig. 5A). This approach revealed decreased costimulatory B7-2A activity in 5 of 12 CHO-B7-2A/C cell lines, as compared with the reciprocal CHO-B7-2A/mock transfectants examined. These five cell lines (three independent experiments for each) were found to display median reductions of costimulatory activity (range) of 69% (58–82%), 34% (31–41%), 23% (21–27%), 39% (24–44%), and 32% (23–33%), respectively (Fig. 5, B and C). The quantitative analysis of the surface expression of B7-2A and B7-2C proteins (Fig. 5D) in the twelve CHO-B7-2A/C transfectants had shown significantly lower surface B7-2A to B7-2C protein expression ratios (median ratio: 1.4, range: 1.0 to 3.5) in those with reduced B7-2A costimulatory function, compared with those with unaffected B7-2A costimulatory activity (median ratio: 14.6, range: 10.0 to 19.5).

Discussion

In this study, we describe a novel alternate transcript of the B7-2 costimulatory molecule, named B7-2C. B7-2C mRNA is generated by the splicing of exon 4 that results to the complete deletion of the IgV-like counter-receptor binding extracellular domain. Similarly to the splice variant that encodes the soluble molecule (11), the open reading frame of the B7-2C transcript is not affected. A truncated variant of the murine B7-2 costimulatory molecule with undefined function has been previously reported (8). In fact, in a manner analogous to human B7-2C, this transcript lacks a segment of the IgV-like counter-receptor binding domain and intriguingly, it was detected in keratinocyte epithelial cells. Splice variants of murine and human B7/CD28 molecules that encode truncated proteins with distinct functions have also been reported (9, 10, 12, 19). B7-1a is a splice isomer of the murine B7-1 protein that lacks the IgC-like protein region and has been implicated in the activation of experienced T cells (10). The truncated variant of the human CD28, which lacks almost the entire extracellular ligand binding region, has been described to associate with the full-length CD28 molecule and to act as an amplifier of the full-length CD28 costimulatory signal (9). The murine ligand-independent CTLA4 isoform that is expressed in memory/regulatory T cells has been found to rapidly up-regulate upon activation and to strongly inhibit T cell responses, whereas its expression has been associated with the development of autoimmune disorders, such as Grave’s disease, autoimmune hypothyroidism, and type 1 diabetes (12).

The truncated B7-2C transcript was initially observed in long-term cultured human non-neoplastic SGECs. In fact, evidence gathered during the last decade, suggest that SGECs possess features of nonprofessional APCs, including the expression of functional cell surface and soluble B7-2 costimulatory molecules (15, 17). Of interest, besides SGECs, B7-2C was found to be expressed by monocytes, which are considered as archetypal APCs, but not by other B7-2A-expressing immune cells, including DCs, the foremost population of APCs, as well as B and T lymphocytes. Intriguingly, in line with previously reported findings (11), our data indicate that DCs solely express the full-length B7-2 isoform. In particular, the differential pattern of B7-2C expression in monocytes, DCs, and B cells is indicative of specific transcriptional regulation of the expression of B7-2 molecules in these types of APCs, which may be supportive of their distinctive functional properties. Further studies need to clarify whether B7-2C expression extends to other types of non-neoplastic epithelial cells, besides SGEC. Several neoplastic epithelial cell lines of different tissue origin were found negative; however, these cell lines were not expressing any of the B7-2 molecules. In addition, none of the B7-2 variants was detected in salivary gland fibroblasts. B7-2C mRNA expression in SGEC was related to low constitutive surface expression of the full-length B7-2A protein, whereas the expression of the full-length B7-2A and the truncated B7-2C protein forms appears to be reversely regulated. In fact, the expression of the surface B7-2C protein by monocytes was found to be downregulated following activation procedures that augment the expression of the B7-2A protein, such as PGN treatment or plastic adhesion. Based on the data shown, it is tempting to hypothesize that B7-2C expression alludes to cells with Ag-presenting capacity and that it plays a role in the regulation of immune responses.

As anticipated by the lack of the IgV-like counter-receptor binding domain, B7-2C protein was found unable to interact with B7-2A counter-receptors. Nevertheless, the coexpression of the truncated B7-2C with the full-length B7-2A was shown to negatively affect the B7-2A costimulatory activity, suggesting that the B7-2C protein inhibits the transmission of B7-2 costimulatory signals to T cells. This effect likely relates to the relative surface expression of the two isoforms, as it was observed in double-transfected CHO-B7-2A/C cell lines with a lower ratio of surface B7-2A to B7-2C protein expression (median ratio: 1.4, range: 1.0 to 3.5) in those with reduced B7-2A costimulatory function, compared with those with unaffected B7-2A costimulatory activity (median ratio: 14.6, range: 10.0 to 19.5).
The presence of three potential sites of protein kinase C phosphorylation in the cytoplasmic tail of B7-2 molecules suggests signaling transmission to expressing cells. In fact, the interaction of B7-2A with counter-receptors on T cells has been shown to act bidirectionally and to create signals to B7-2A-expressing APCs (21–25). In this context, the conservation of the B7-2A cytoplasmic tail in the B7-2C isoform may indicate a signaling role of B7-2C molecules in the B7-2A/B7-2C-expressing cells, possibly as a modulator of the B7-2A signaling.

In conclusion, our findings suggest that B7-2C represents an additional negative regulatory mechanism, which participates in the fine tuning of T cell responses. Although further in vivo studies are needed to elucidate the physiological role of the B7-2C isoform, B7-2C may act by attenuating the transmission of positive costimulatory signals to T cells by B7-2A molecules constitutively expressed on resting classical and nonprofessional APCs. Nevertheless, the occurrence of the B7-2C splice variant further indicates the complexity and the tight regulatory mechanisms that control the fate of T cell immune responses.

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References


